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(54) Title: A PROCESS OF PRODUCING EXTRACELLULAR PROTEINS IN BACTERIA

(57) Abstract

The invention relates to a method of producing an extracellular protein in a bacterium provided with an inner and an outer cell membrane, the method comprising: (a) providing a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein, (b) transforming cells of a microorganism provided with an inner and outer cell membrane with the recombinant vector of step (a), (c) culturing the transformed cells of step (b) under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein into the culture medium, and (d) recovering the resulting protein from the medium.

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A PROCESS OF PRODUCING EXTRACELLULAR PROTEINS IN BACTERIA

FIELD OF INVENTION

The present invention relates to a method of producing an extracellular protein in a bacterium, as well as to a DNA construct and a recombinant vector comprising a DNA sequence encoding said protein.

BACKGROUND OF THE INVENTION

Prokaryotic organisms provided with both an inner and outer cell membrane such as gram-negative bacteria only rarely secrete proteins out of the cell into the surrounding medium. Such proteins which do not remain in the cytoplasm are usually exported across the cytoplasmic membrane into the periplasmic space but do not cross the outer cell membrane. However, more recently examples have been found of proteins which are truly secreted from gram-negative bacteria.

Thus, *Lysobacter enzymogenes* secretes an alkaline phosphatase (cf. S. Au et al., J. Bacteriol. 173(15), 1991, pp. 4551-4557) and an α-lytic protease. Attempts to express the α-lytic protease in *E. coli* have resulted in production of the enzyme within cells as well as in the extracellular medium (cf. J.L. Silen et al., J. Bacteriol. 171(3), 1989, pp. 1320-1325).

Likewise, *Achromobacter lyticus* produces an extracellular protease, the primary structure of which appears from S. Tsunasawa et al., J. Biol. Chem. 264(7), 1989, pp. 3832-3839. The gene encoding *A. lyticus* protease I was cloned in *E. coli* in which the enzyme was exported to the periplasm rather than secreted into the culture medium (cf. T. Ohara et al., J. Biol. Chem. 264(34), 1989, pp. 20625-20631).

SUMMARY OF THE INVENTION

It has now been found possible to obtain extracellular production of proteins from a heterologous host bacterium which does not readily translocate proteins out of the cells. Such extracellular production is accomplished by means of a prepropeptide or part of a prepropeptide of certain bacterial extracellular proteases.

Accordingly, the present invention relates to a method of producing an extracellular protein in a bacterium provided with an inner and outer cell membrane, the method comprising

(a) providing a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein,

(b) transforming cells of a bacterium provided with an inner and outer cell membrane with the recombinant vector of step (a),

(c) culturing the transformed cells of step (b) under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein into the culture medium, and

(d) recovering the resulting protein from the medium.

In the present context, the term "bacterium provided with an inner and outer cell membrane" is intended to indicate a bacterium which has an inner, or cytoplasmic, membrane surrounding the cytoplasm of the cell as well as an outer membrane and a periplasmic space between the inner and outer membrane. In

most such organisms, there are mechanisms (including signal sequences) permitting the translocation of expressed gene products across the inner membrane to the periplasm, while secretion of proteins through the outer membrane is far less common. The term "heterologous", when applied to host cells, is intended to indicate that the host cell is one which does not, in nature, produce the protein in question.

The term "prepropeptide" is intended to indicate a peptide composed of a signal peptide (the prepeptide) and one or more peptide sequences present on a precursor form of the protein to be produced. If a part (or fragment) of a prepropeptide is employed in the method of the invention, it should be sufficient in length to have retained the ability of the full-length prepropeptide to bring about extracellular production of the protein of interest.

The term "bacterial extracellular protease" is intended to indicate a proteolytic enzyme produced in bacteria and secreted from bacterial cells. Examples of suitable bacterial extracellular proteases are *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases, such as subtilisins.

The term "operably connected" is intended to indicate that the DNA sequence encoding the prepropeptide is transcribed together with the DNA sequence encoding the desired protein.

The protein produced by the present method may be either homologous or heterologous, either to the prepropeptide or to the host cell or both. Thus, it may be envisaged that the DNA sequence encoding the protein of interest may be preceded by a DNA sequence encoding a prepropeptide which, in nature, is connected to the protein-coding DNA sequence expressed in a host bacterium which does not naturally produce the protein. Alternatively, the DNA sequence encoding the protein of interest may be preceded by a DNA sequence encoding a prepropeptide which is not naturally connected to the protein-coding DNA

sequence expressed in a host bacterium which produces the protein in nature. Furthermore, the DNA sequence encoding the protein of interest may be preceded by a DNA sequence encoding a prepropeptide which is not naturally connected to the protein-coding DNA sequence expressed in a host bacterium which does not naturally produce the protein.

The term "leakage" is intended to indicate that the protein produced by the present method is transported out of the cell either by secretion, i.e. translocation across both the inner and outer cell membrane, or by export of the protein to the periplasm followed by lysis of the outer membrane. The lysis of the outer membrane may be complete or partial, or the protein produced by the present method is transported out of the cell by export of the protein to the periplasm followed by release through the outer cell membrane.

In another aspect, the present invention relates to a method of producing an extracellular protein in a bacterium provided with an inner and outer cell membrane, in which method a bacterium provided with an inner and outer cell membrane and transformed with a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein, is cultured under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein into the culture medium, and the resulting protein is recovered from the medium.

In a further aspect, the present invention relates to a recombinant expression vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus*

metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein. The vector is useful for transformation of a suitable host microorganism in the method of the invention described above.

In a still further aspect, the present invention relates to a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein. The DNA construct may suitably be inserted into a recombinant vector as indicated above.

DETAILED DESCRIPTION OF THE INVENTION

In the *Acromobacter lyticus* genome, the gene coding for *A. lyticus* protease I encodes a polypeptide of 653 amino acid residues. This includes a signal (or pre) peptide of 20 amino acids, an N-terminal propeptide of 185 amino acids, a core protein of 268 amino acids which is the active protease, and a C-terminal propeptide of 180 amino acids, as shown in Fig. 11 (cf. T. Ohara et al., J. Biol. Chem. 264, 1989, pp. 20625-20630).

In a preferred embodiment of the invention, the DNA construct comprises a DNA sequence encoding the prepeptide and a 185 amino acid N-terminal propeptide, but not the 180 amino acid C-terminal propeptide, of *A. lyticus* protease I. It has surprisingly been found possible to omit the C-terminal propeptide while obtaining extracellular production of the desired protein. Omission of the C-terminal propeptide results in the formation of a homogeneous extracellular product which is, for instance, easier to purify than the heterogeneous product obtained when the C-terminal propeptide is present. The DNA construct may further comprise a DNA sequence encoding at least part of the *A. lyticus* protease I, as such a

sequence has been found to facilitate transport of the desired protein out of the host cell. If such a DNA sequence is included in the construct, it may encode full-length *A. lyticus* protease I core protein. The protease may either be in active form, or it may be inactivated, e.g. by deletion of one or more amino acids at the C-terminal end of the protease, or by substitution of one or more of the amino acids of the catalytic triad (His57, Asp113 and Ser194). For example, Ser194 may suitably be substituted by Ala.

Examples of desired proteins are *A. lyticus* protease I core protein, glucagon-like peptide-1, growth hormone, tissue factor pathway inhibitor, aprotinin, trypsin, insulin or an insulin precursor or analogue, or enzymes such as lipases, amylases, cellulases or proteases.

The DNA construct of the invention encoding the desired protein may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., supra).

The DNA construct of the invention encoding the protein may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments

corresponding to various parts of the entire nucleic acid construct, in accordance with standard techniques.

The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in PCR Protocols, 1990, Academic Press, San Diego, California, USA. For instance, it may be envisaged that the DNA sequence encoding the prepropeptide may be prepared by PCR amplification of chromosomal DNA of the species from which the prepropeptide is derived. Likewise, the DNA sequence encoding the desired protein may be prepared by PCR amplification of chromosomal DNA from the species from which the protein is derived, or for instance by screening a genomic or cDNA library with oligonucleotides as indicated above.

The recombinant vector into which the DNA construct of the invention is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the desired protein is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the protein.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The promoter is preferably an inducible promoter, and more particularly one where expression from the promoter may be turned off by a repressor under non-induced conditions. Examples of inducible promoters include the phage Lambda P_R or P_L promoters or the E. coli lac, trp or tac promoters.

The vector may also comprise a selectable marker, e.g. a gene the product of which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin or chloramphenicol.

The procedures used to ligate the DNA sequences coding for the desired protein, the promoter and preprosequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA).

The bacterial host cell into which the DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of extracellularly producing the desired protein and includes gram-negative bacteria such as *Echerichia coli* or *Pseudomonas*. The transformation of the bacteria may be effected by protoplast transformation or by using competent cells in a manner known per se (cf. Sambrook et al., supra).

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The protein produced by the cells may then

be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of protein in question.

The protein recovered in step (d) of the method of the invention may be in precursor form, i.e. it is recovered as a polypeptide comprising the propeptide fused to the protein. In this case, the precursor may subsequently be subjected to maturation, in particular by enzymatic processing procedures known *per se* in the art. The enzyme used for maturation is usually so selected that it is is specific for the amino acid residue(s) at the desired cleavage site. Examples of such enzymes are trypsin, trypsin-like protease derived from *Fusarium oxysporum* (WO 89/6270), clostripain (W.M. Mitchell et al., Methods Enzymol. 19, 1970, p. 635), mouse submaxillary gland protease (M. Levy et al., Methods Enzymol. 19, 1970, p. 672), thrombin or other proteolytic enzymes of the blood coagulation cascade (e.g. Factor Xa), bovine enterokinase, *Staphylococcus aureus* V8 protease, or *Bacillus licheniformis* Glu/Asp-specific serine protease. If no appropriate cleavage site is present in the precursor polypeptide, it may be provided, e.g. by site-directed mutagenesis of the DNA sequence coding for the precursor to introduce one or more codons specifying the desired amino acid residue(s).

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

Example 1:

Based on the published sequence of the gene coding for the *Achromobacter lyticus* protease I (Ohara et al. (1989), J. Biol. Chem **264**, 20625-20631) PCR

primers were synthetizised in order to amplify either the first half, the second half, or the whole gene when using *Achromobacter lyticus* M497-1 chromosomal DNA as template in the PCR reactions.

Chromosomal DNA was prepared from *Achromobacter lyticus* M-·-97-1 following the procedure of Kamelendu Nath (Nucleic Acids Res. **18**(21), 1990, p. 6442) with a start volume of 50 ml over night LB culture.

A fragment of the gene was amplified by using the two primers:

MHJ783: 5'-AAAAACTGCAGCGCTCGCCGCCCCGGCCTCGC-3'
(introduces a Pst I site in the signal sequence coding part of the gene)

MHJ782: 5'-AAAAAGGTACCGGTCGCGACGGTCCCAACCGGCCC-3'
(introduces a Kpn I site in the middle part of the region coding for the mature enzyme)

Reaction mixture:

10 µl 10 X PCR buffer
8 µl 2.5 mM dNTP
10 µl 10 pmol/µl primer MHJ783
10 µl 10 pmol/µl primer MHJ782
0.5 µg *A. lyticus* M497-1 DNA
59.5 µl H_2O
o.5 µl Taq polymerase (added at 95°C)

The reaction was run for 30 cycles under the following conditions:

Denaturing	95°C	1 min
Annealing	72°C	1 min

Polymerization	72°C	2 min

5 µl of the reaction mixture was run on a 1 % agarose gel and a band of the right size (997 bp) was observed.

The DNA could be digested with Asc I, Not I, Sal I, and Xho I as was expected for the protease gene.

50 µl of the reaction mixture was digested with Pst I and Kpn I, isolated from an agarose gel and ligated into pUC19 (C. Yanisch-Perron et al., Gene 33, 1985, pp. 103-119) digested with the same enzymes. The ligation mixture was transformed into *E. coli* 803-9 and plasmid was prepared.

In this manner, the first half of the gene was amplified. This was cloned into pUC19 using the Pst I and Asp I sites incorporated in the ends of the primers. Sequencing revealed that it was the correct DNA that had been cloned, and the fragment was labelled using random hexamer primers with α-^{32}P-dATP whereupon a Southern hybridization was performed on *A. lyticus* chromosomal DNA cut with various restriction endonucleases. This indicated that the gene was situated on an approximately 2.1kb SphI - Nco I fragment.

A library was made from Sph I - Nco I digested *A. lyticus* DNA of approximately 2100 bp in size and cloned into Sph I- Nco I digested pSX54 (a pUC18 (Yanisch-Perron et al., *supra*) derived cloning vector). Around 10.000 colonies on 6 plates were lifted onto Whatman 540 ashless filters and hybridized at 65°C with the probe described above (Sambrook el al. (1989), Molecular Cloning, Cold Harbor Laboratory Press). The filters were washed at the same temperature and placed onto X-ray films. After exposure it turned out that about 1 % of the colonies were positive. 25 were reisolated and plasmids were prepared. These were cut with Pst I and EcoRI and 3 turned out to have the expected restriction pattern.

One of these (pSX494, fig. 1) was digested with Nco I, filled out with Klenow polymerase and the four dNTP's, and digested with Sph I. The 2.1kb band was isolated from an agarose gel and ligated into dephosphorylated Sph I - Sma I digested pUC19 giving rise to pSX512 (fig. 2). The plasmid was transformed into *E. coli* W3110 lacI^q (*E. coli* W3110 is an early isolate and has been used extensively as ancestral stock for the K-12 strain (B. Bachman, Bacteriol. Rev. 36, 1972). The W3110 strain used in the present method has been made *lacI^q* in order to overproduce the Lac repressor, turning off expression from p*lac* more completely). The resulting strain was shown to hydrolyse Z-lys (Benzoyl-lysyl-pNA) when induced with either IPTG or lactose. Western blot analysis (with antibodies raised against Achap I available from WAKO Co.) showed that the induced strain produced a protein that was too big compared to the known enzyme.

Example 2:

A new DNA construct was made wherein the 3'-end of the coding region was deleted meaning that the resulting protein would lack the C-terminal extension that is cleaved off in the original *A. lyticus* strain. Two UGA stop codons and a Hind III site was introduced by PCR in the *A. lyticus* gene at the site where the portion of the gene coding for the mature enzyme ends. This Hind III site was subsequently filled out with Klenow polymerase and ligated to the Asp 718 I site (also filled out) on pSX512 resulting in pSX547 (fig. 3). This plasmid was transformed into *E. coli* W3110 lacI^q and plated onto LB-plates with 200 µg/ml ampicillin (J. H. Miller (1972), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory).

The resulting strain was grown in liquid LB medium containing 0.4% lactose for 44 hours at 26°C whereafter the culture was centrifuged and the supernatant tested for lysyl -endopeptidase activity with Benzoyl-lysyl-pNA. The result was positive and a Western blot analysis (see above) showed that the enzyme

produced from this strain had exactly the same size as the commercially available product (Achap I). It was also shown that the enzyme had the same specific activity.

Example 3.

A construct was made where the *Humicola insolens* lipase gene was fused to the Savinase (Subtilisin 309) signal sequence and the expression was under control by xylose:

pSX92 (WO 89/06279) was cut with Hind III, blunt ended with Klenow polymerase (- nucleotides), and then cut with Cla I. The large fragment was isolated (A).

pHLL (see EP 305,216 figures 3 and 4) was cut with Bam HI, blunt ended like above, and then cut with Xho II. The fragment containing the mature part of the lipase gene was isolated (B, 818bp.)

A and B were ligated together with a synthetic linker which codes for the last 5 amino acids in the Savinase signal fused to the first four amino acids of the mature lipase (fig. 4a). The last A in the upper strand changes the Xho II site in the lipase gene to a Bgl II site:

```
KFN 575/576:   5'- CG ATC GCA TCG GCT GCT GAG GTC TCG CAA -3'
                 3'-  TAG CGT AGC CGA CGA CTC CAG AGC GTT CTAG -5'
                          Savinase       |      lipase
```

The resulting plasmid pSX167 was cut with Pme I and Bam HI and the fragment containing the Savinase-Lipase fusion and the 5S terminator was isolated (1769 bp.). This fragment was ligated into Hinc II - Bam HI cut pUC19 (Yanish-Perron et al. (1985) GENE 33, 103-119) giving rise to pSX578 (fig 4b).

The prepro-part of the *A. lyticus* protease gene was fused to the lipase using the PCR technique "splicing by overlap extension" described by Horton et al. (1989), GENE 77, 61-68.

Primers for the *A. lyticus* protease I prepre- mature *H. insolens* lipase fusion:

MHJ 3799: 5' - ACT CGG CGC GCC AAC TGT GGA CGG-3'
Asc I

MHJ 3852:

5' -GAC CTC TTT ATC ATC GTC GTC CTT CTC GCC GGA CGC AGC GGC CAG GC-3'
lipase ¦ Enterokinase site ¦ pro-A. *lyticus* protease

MHJ 3829: 5' -GAC GAC GAT GAT AAA GAG GTC TCG CAA GAT CTG TTT AAC C-3'
Enterokinase site ¦ mature lipase

MHJ 3800: 5' - CCA GAT TTG ATC CAG TAC TCT GGG C-3'
Bst XI

Two PCR products were made: MHJ 3799/ MHJ 3852 with pSX547 as template and MHJ 3829/ MHJ 3800 with pSX578 as template. These two products were mixed, denatured and a joined PCR product was made with MHJ 3799 and MHJ 3800 as primers. This was cut with Asc I and Bst XI and used in the construction of pSX579 (fig. 4c). This plasmid was transformed into *E. coli* W3110 lacI^q and the strain was cultivated as described in example 2.

Example 4.

The prepro-part of the *B. subtilis* Savinase gene (Subtilisin 309) was fused to the mature part of the lipase gene in the same way as above using the following primers:

MHJ 3790: 5' -AAA AAA GCT TGG AGA AAC CGA ATG AAG AAA C-3'
Hin dIII ¦ Start Savinase

MHJ 3851:

5' -GAC CTC TTT ATC ATC GTC GTC CAT TGT CGT TAC TTC TGC ATC CTC-3'
lipase ¦ Enterokinase site ¦ Pro- Savinase

A PCR product was made using these primers and pSX92 as template (MHJ 3790 introduces a Hind III site right at the ribosome binding site in front of the Savinase initiation codon). This was mixed with the MHJ 3829/ MHJ 3800

product from above, denatured and a joined product was made with MHJ 3790 and MHJ 3800 as primers. This was cut with Hind III and Bst XI and used in the construction of pSX580 (fig. 4d). This plasmid was transformed into *E. coli* W3110 lacI^q and the strain was cultivated as described in example 2.

Example 5 :

Expression in *E.coli* of glucagon-like peptide I fused to *A.lyticus* protease I prepro-region.

The construction of expression vectors for glucagon-like peptide I (GLP-1) fused to *A. lyticus* alkaline protease I (Achap I) prepro-region, is outlined in Figs. 5-6. Oligonucleotide linkers coding for the protease signal peptide and the first 29 amino acids in the pro-region have the following sequence :

```
2318: 5' CGATGAAACGTATCTGCGGATCCCTGCTGCTGCTGGGTCT
2317: 3'   TACTTTGCATAGACGCCTAGGGACGACGACGACCCAGA

      GAGCATCAGCGCGGCGCTGGCGGCGCCGG     3'      2318
      CTCGTAGTCGCGCCGCGACCGCCGCGGCCGATC 5'      2317
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2316 :  5' CTAGCCGTCCGGCGGCGTTCGATTATGCGAACCTGAGCAGCGTGGA
2315 :  3'     GGCAGGCCGCCGCAAGCTAATACGCTTGGACTCGTCGCACCT

        TAAAGTGGCGCTGCGTACCATGCCGGCGG     3'      2316
        ATTTCACCGCGACGCATGGTACGGCCGCCAGCT 5'      2315
```

The linker sequences, which are optimized for best codon usage in *E.coli,* were subcloned into the versatile vector pHD 414 (described in WO 92/16634), cut with Cla1 and Sal1. The C-terminal part of the pro-region from amino acid 30-185 was subcloned into pUC19 Sal1-BamH1 as described using pSX 512 (Fig.2) for PCR priming with :

```
2590 :   5'      CAGCAACAACAACTCGGCGCGCC        3'
2591 :   3'      GCAGGCCTCTCTTCCCGCACAGC         5'
                   *
```

and the oligonucleotide linkers :

```
2592 :    5'       CCGGAGAACGTG                3'
2593 :    3'            TCTTGCACCTAG           5'
```

At position 185 the lysine residue has been substituted with arginine. A* is introduced in-stead of C in 2591 to create a BspE1 site.

The expression system shown in Fig.6 is pUC19 containing the *lac* promoter. A Hind3-Cla1 linker, which is also adapted for the Tet promoter, was subcloned into pUC19 using a Cla1-BamH1 spacer fragment from pBR322. The linker sequences are as follows :

```
2737 :   5'     AGCTTTAATGCGGTAGTTTATCACAGTTAAATTG      3'
2747 :   3'         AATTACGCCATCAAATAGTGTCAA            5'

2748 :   5'           CTAACGCTTAAGGAGGTTAAT             3'
2738 :   3'     TTTAACGATTGCGAATTCCTCCAATTAGC           5'
```

The promoter and linker were joined to the Achap I prepro-region in pHW1163, in which the GLP-1 gene was inserted as a BamH1-EcoRV fragment of 250bp and 550bp, respec-tively, corresponding to a monomer and a tetramer of the gene (prepared synthetically as described in DK 1440/93). The resulting expression plasmids pHW1166 and pHW1167 were transformed into *E.coli* W3110 $lacI^q$ and GLP-1 expression was measured in supernatants by Western blot analysis using specific antibodies. Processed as well as unprocessed material was detected in the supernatants.

Example 6 :

Expression in *E.coli* of GLP-1 fused to *A.lyticus* protease pre-pro-core region inactivated by truncation.

The core region codes for the mature enzyme, which can be inactivated by truncation. This has been done in two ways as shown in Fig.7. Using a Bsg1-BamH1 linker :

```
2382 : 5'    GTGTGACCGAACCGGGTGTGCTGGGTCAGCGTG          3'
2381: 3'  GCCACACTGGCTTGGCCCACACGACCCAGTCGCACCTAG      5'
```

the core region is deleted of 60 C-terminal amino acids and an additional deletion of 12 amino acids including serine in the catalytic triad as shown in Fig.10.

Using a Pst1-BamH1 linker :

```
2329: 5'          GCGCGACCGGCACCAACCGTG            3'
2327: 3'      ACGTCGCGCTGGCCGTGGTTGGCACCTAG        5'
```

the core region is deleted of 45 C-terminal amino acids, just leaving the possibility of creating all three sulphur bridges found in the native core.

The linkers were joined to most of the Achap I gene starting from Sal1-site as described before to create pHW1158 and pHW1159. From there on the procedure to add promoter, N-terminal part of the Achap I gene and GLP-1 gene as monomer and as tetramer, is shown in Fig.8. It follows the same pattern as described in Fig.6 for the prepro-con-structions. The expression plasmids pHW1168-1171 were analyzed as described in Example 5. Processed and unprocessed material was detected in the culture supernatants.

Example 7 :

Expression in *E.coli* of GLP-1 fused to *A.lyticus* protease prepro-core region inactivated by mutation.

Mutation without deletion is probably the best way to mimic the native situation as for folding of the core region and still prevent catalysis of the product. We have chosen to mutate the active serine residue to alanine by introducing a PCR fragment C-terminally, using pSX512 as template and the following primers :

```
3319 :    5'    CATTTGACCGTGCAGTGGCAGCCCTCGGGCGGCGTGACCGAGCC
                GGGTTCGGCGGGTTCG              3'

3320 :    3'    GTCAAGTAGCTACCGGACCTAAGCCCGCCGCCGTGCGGCGCGC
                CTAGGCGGCCACCGCTT             5'
```

The procedure is depicted in Fig.9. The resulting plasmids pHW1172 containing the GLP-1 monomer and pHW1173 containing the GLP-1 tetramer were analysed as described with the other constructions. Processed and unprocessed material was detected in the culture supernatants.

All the constructions of examples 5-7 are summarized in Fig.10.

CLAIMS

1. A method of producing an extracellular protein in a bacterium provided with an inner and outer cell membrane, the method comprising

(a) providing a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein,

(b) transforming cells of a microorganism provided with an inner and outer cell membrane with the recombinant vector of step (a),

(c) culturing the transformed cells of step (b) under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein into the culture medium, and

(d) recovering the resulting protein from the medium.

2. A method according to claim 1, wherein the bacterial extracellular protease propeptide fused to the desired protein is transported out of the cell by secretion, i.e. translocation across both the inner and outer cell membrane.

3. A method according to claim 1, wherein the bacterial extracellular protease propeptide fused to the desired protein is transported out of the cell by export of the protein to the periplasm followed by lysis of the outer membrane.

4. A method according to claim 3, wherein the lysis of the outer membrane is a partial lysis.

5. A method according to claim 1, wherein the bacterial extracellular protease propeptide fused to the desired protein is transported out of the cell by export of the protein to the periplasm followed by release from the outer cell membrane.

6. A method according to any one of the preceding claims, wherein the DNA construct comprises a DNA sequence encoding the prepeptide and N-terminal propeptide, but not the C-terminal propeptide, of *A. lyticus* protease I.

7. A method according to claim 6, wherein the DNA construct further comprises a DNA sequence encoding at least part of the *A. lyticus* protease I.

8. A method according to claim 7, wherein the DNA construct comprises a DNA sequence encoding full-length *A. lyticus* protease I core protein.

9. A method according to claim 8, wherein the *A. lyticus* protease I is in active form.

10. A method according to claim 9, wherein the *A. lyticus* protease I is in inactive form.

11. A method according to any one of claim 1, 2, 3, 4, or 5, wherein the DNA sequence encoding the desired protein codes for *A. lyticus* protease I core protein, glucagon-like peptide-1, growth hormone, tissue factor pathway inhibitor, aprotinin, trypsin, or insulin or an insulin precursor or analogue.

12. A method according to any one of claim 1, 2, 3, 4, or 5, wherein the bacterium is a gram-negative bacterium.

13. A method according to claim 12, wherein the gram-negative bacterium is *Escherichia coli*.

14. A method according to any one of claim 1, 2, 3, 4, or 5, wherein the extracellularly produced protein is subjected to a maturation procedure.

15. A recombinant expression vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein.

16. A vector according to claim 15, wherein the DNA construct comprises a DNA sequence encoding the prepeptide and N-terminal propeptide, but not the C-terminal propeptide, of *A. lyticus* protease I.

17. A vector according to claim 16, wherein the DNA construct further comprises a DNA sequence encoding at least part of the *A. lyticus* protease I.

18. A vector according to claim 17, wherein the DNA construct comprises a DNA sequence encoding full-length *A. lyticus* protease I core protein.

19. A vector according to claim 15, wherein the DNA sequence encoding the desired protein codes for *Achromobacter lyticus* protease I core protein, glucagon-like peptide-1, growth hormone, tissue factor pathway inhibitor, aprotinin, trypsin, or insulin or an insulin precursor or analogue.

20. A DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein.

21. A DNA construct according to claim 20, which comprises a DNA sequence encoding the prepeptide and N-terminal propeptide, but not the C-terminal propeptide, of *A. lyticus* protease I.

22. A DNA construct according to claim 21, which further comprises a DNA sequence encoding at least part of the *A. lyticus* protease I.

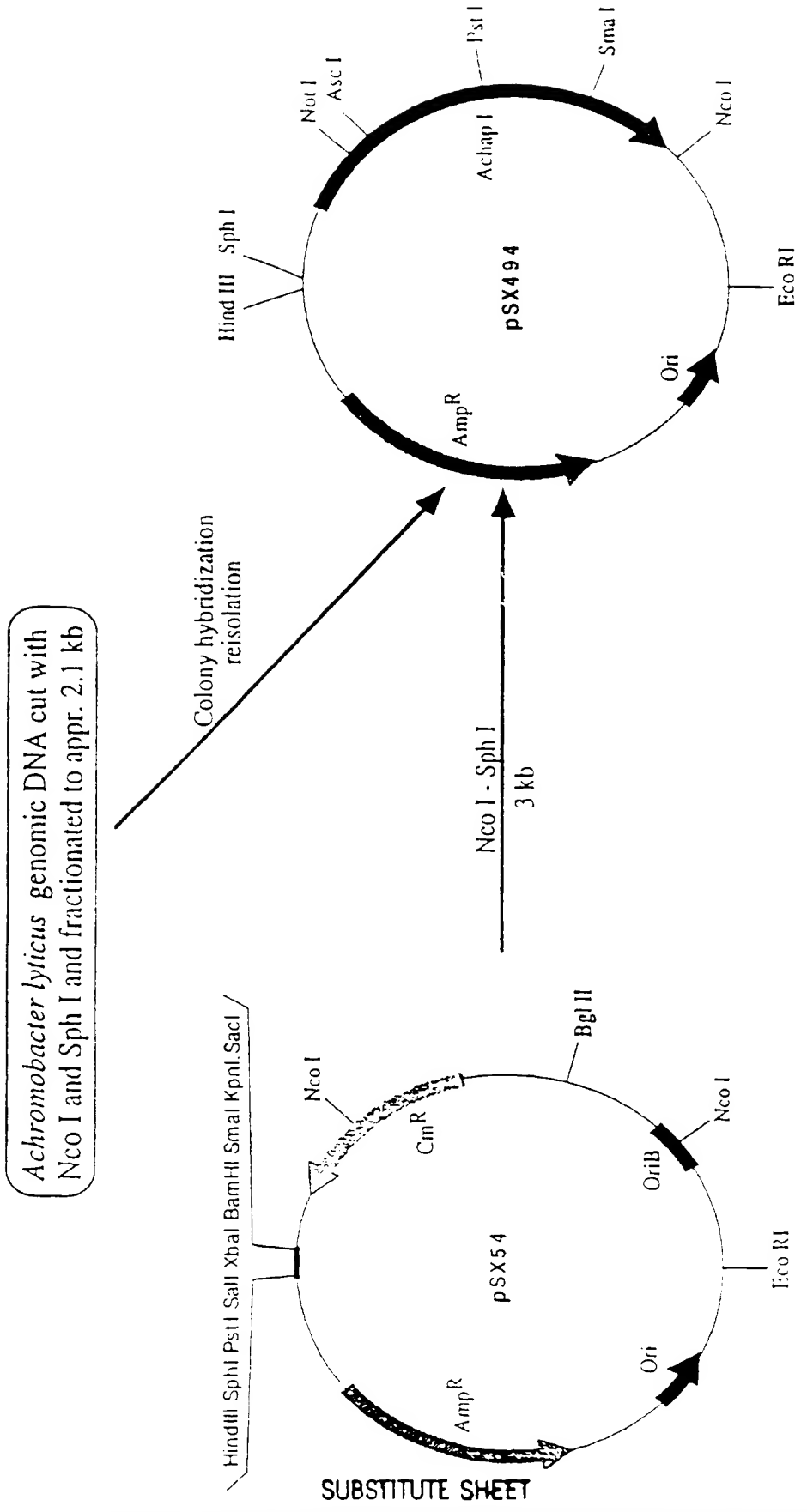
23. A DNA construct according to claim 22, which comprises a DNA sequence encoding full-length *A. lyticus* protease I core protein.

24. A DNA construct according to claim 20, wherein the DNA sequence encoding the desired protein codes for *A. lyticus* protease I core protein, glucagon-like peptide-1, growth hormone, tissue factor pathway inhibitor, aprotinin, trypsin, or insulin or an insulin precursor or analogue.

25. A method of producing an extracellular protein in a bacterium provided with an inner and outer cell membrane, in which method a bacterium provided with an inner and outer cell membrane is transformed with a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein, is cultured under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein into the culture medium, and the resulting protein is recovered from the medium.

26. A method of producing an extracellular protein in a bacterium provided with an inner and outer cell membrane, in which method a bacterium provided with an inner and outer cell membrane is transformed with a recombinant vector including a DNA construct comprising a DNA sequence encoding the prepropeptide or part

of the prepropeptide of a bacterial extracellular protease selected from the group consisting of *Achromobacter lyticus* protease I, *Bacillus* metalloproteases and *Bacillus* serine proteases preceding and operably connected to a DNA sequence encoding a desired protein, is cultured under conditions permitting expression of said DNA insert and leakage of the bacterial extracellular protease propeptide fused to the desired protein through transport out of the cell either by secretion, i.e. translocation across both the inner and outer cell membrane, or by export of the protein to the periplasm followed by partial lysis of the outer membrane or release from the outer membrane into the culture medium, and the resulting protein is recovered from the medium.


Achromobacter lyticus genomic DNA cut with Nco I and Sph I and fractionated to appr. 2.1 kb
Colony hybridization reisolation
Nco I - Sph I
3 kb
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Not I
Asc I
Pst I
Sma I
Nco I
Achap I
Hind III
Sph I
Eco RI
Ori
AmpR
pSX54
Nco I
Bgl II
CmR
OriB
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Eco RI
Ori
AmpR
HindIII SphI PstI SalI XbaI BamHI SmaI KpnI SacI

FIGURE 1

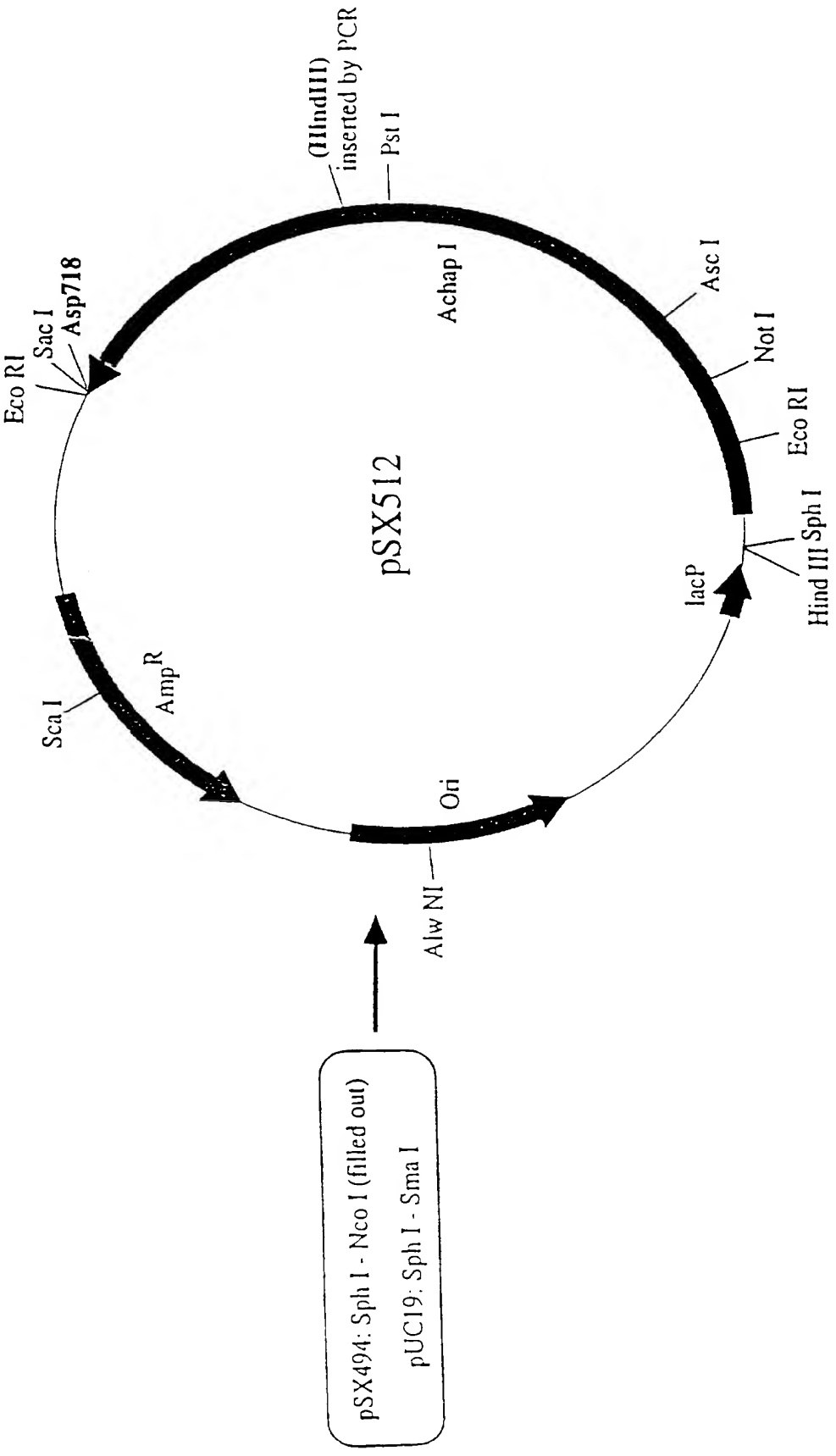
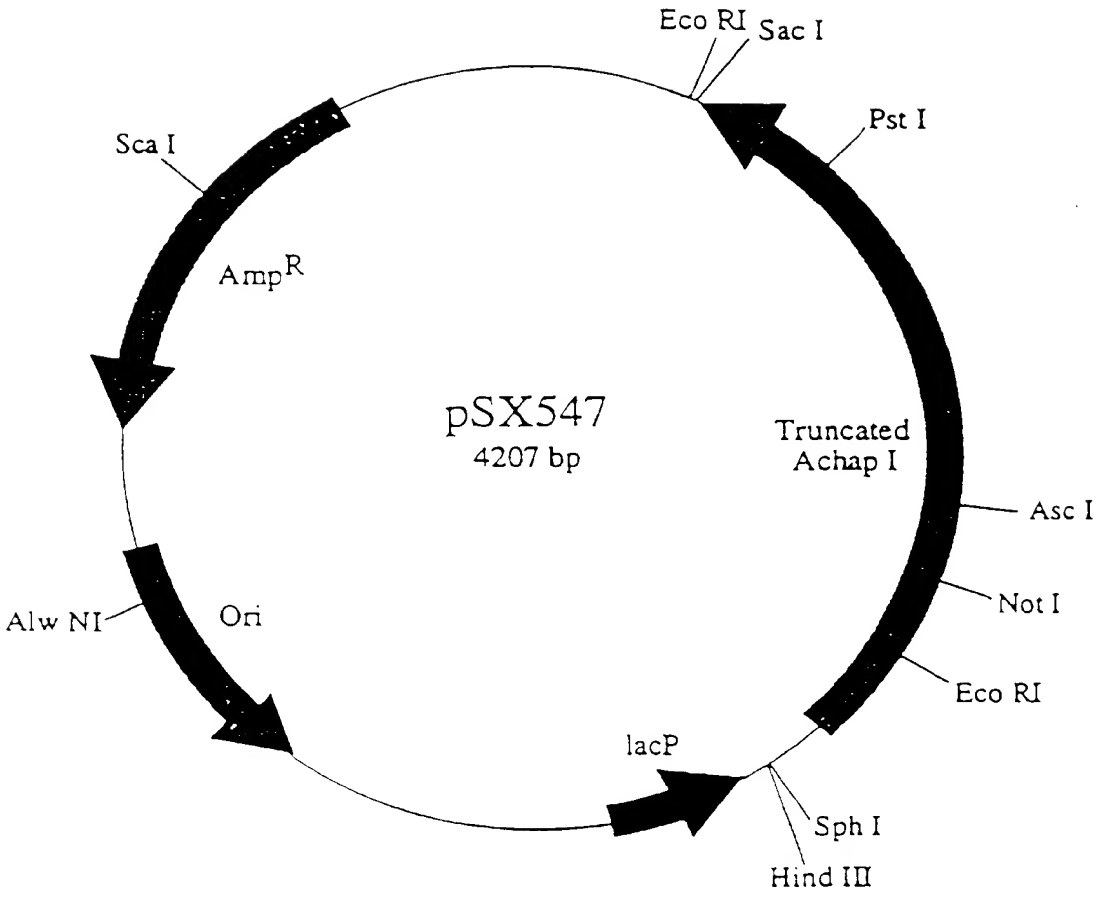
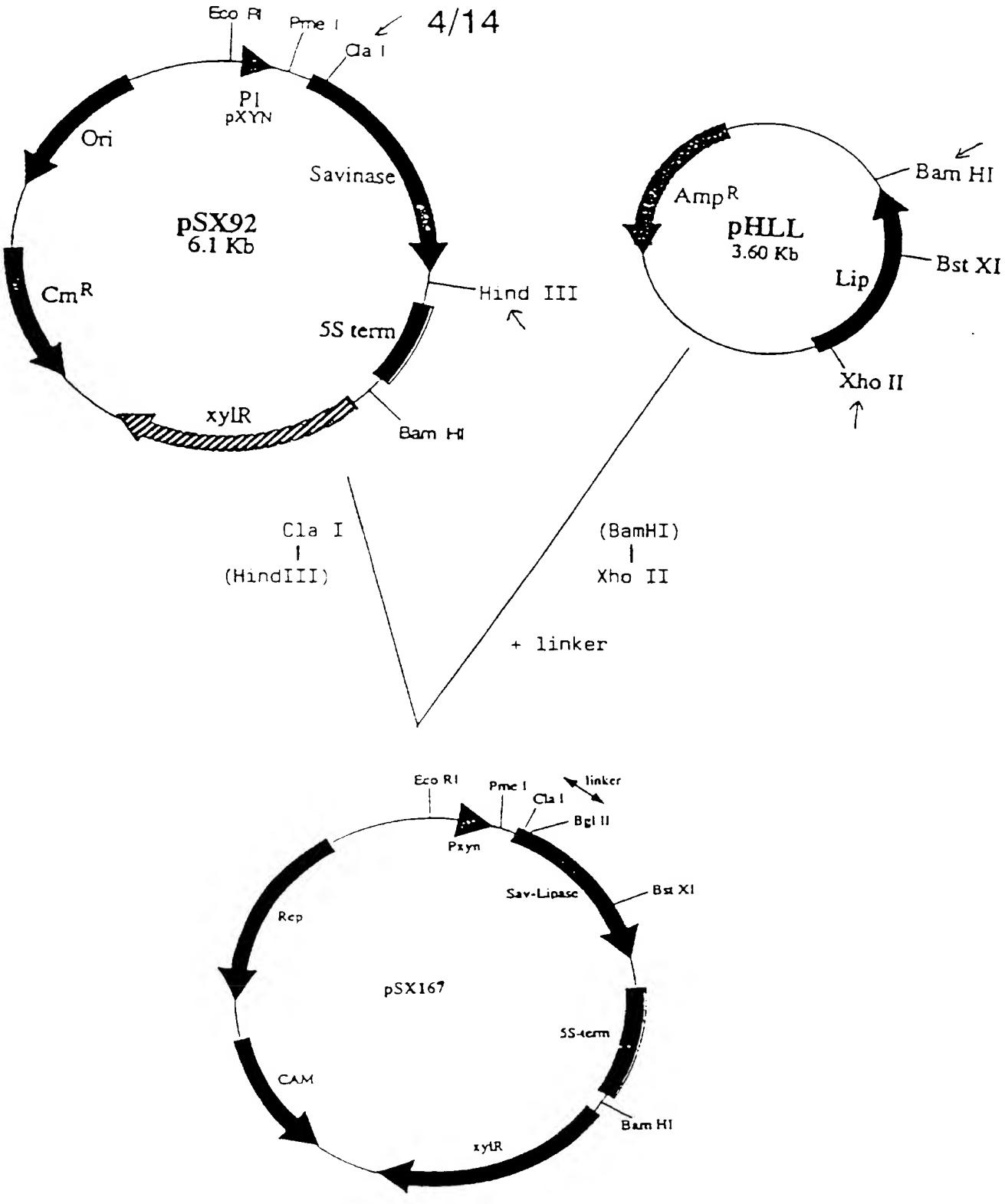

(HindIII)
inserted by PCR
Pst I
Achap I
Asc I
Not I
Eco RI
Hind III
Sph I
lacP
Ori
Alw NI
Sca I
Amp^R
Eco RI
Sac I
Asp718
pSX512
pSX494: Sph I - Nco I (filled out)
pUC19: Sph I - Sma I

FIGURE 2.



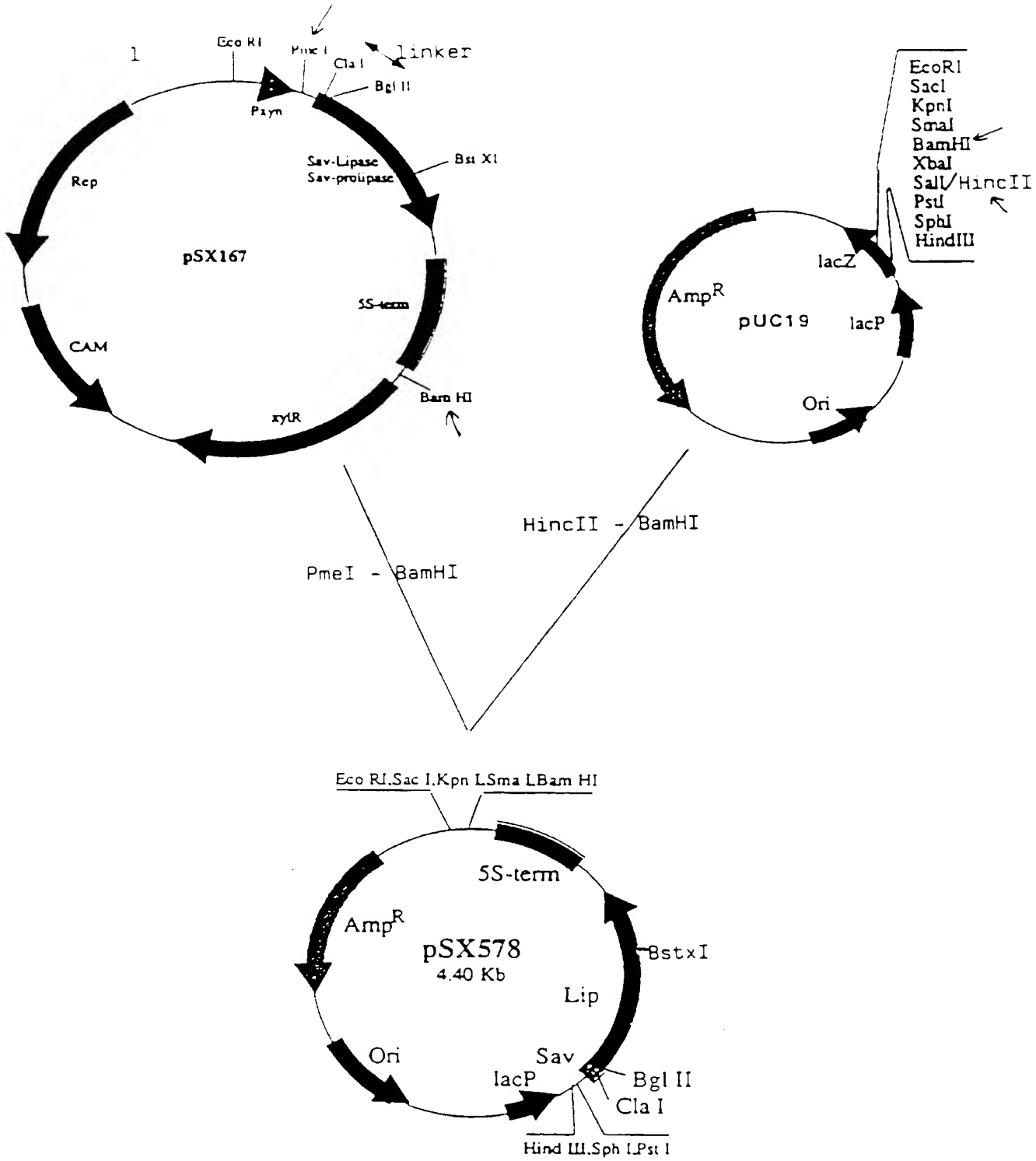
Hind III - Asp 718 deletion of pSX512

FIGURE 3.



Linker: pSX167: KFN 575/576

Fig. 4a


1
Eco RI
Pme I
Cla I
linker
Bgl II
Pxyn
Sav-Lipase
Sav-prolipase
Bst XI
Rep
pSX167
5S-term
CAM
xylR
Bam HI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI/HincII
PstI
SphI
HindIII
lacZ
AmpR
pUC19
lacP
Ori
HincII - BamHI
PmeI - BamHI
Eco RI.Sac I.Kpn I.Sma I.Bam HI
5S-term
AmpR
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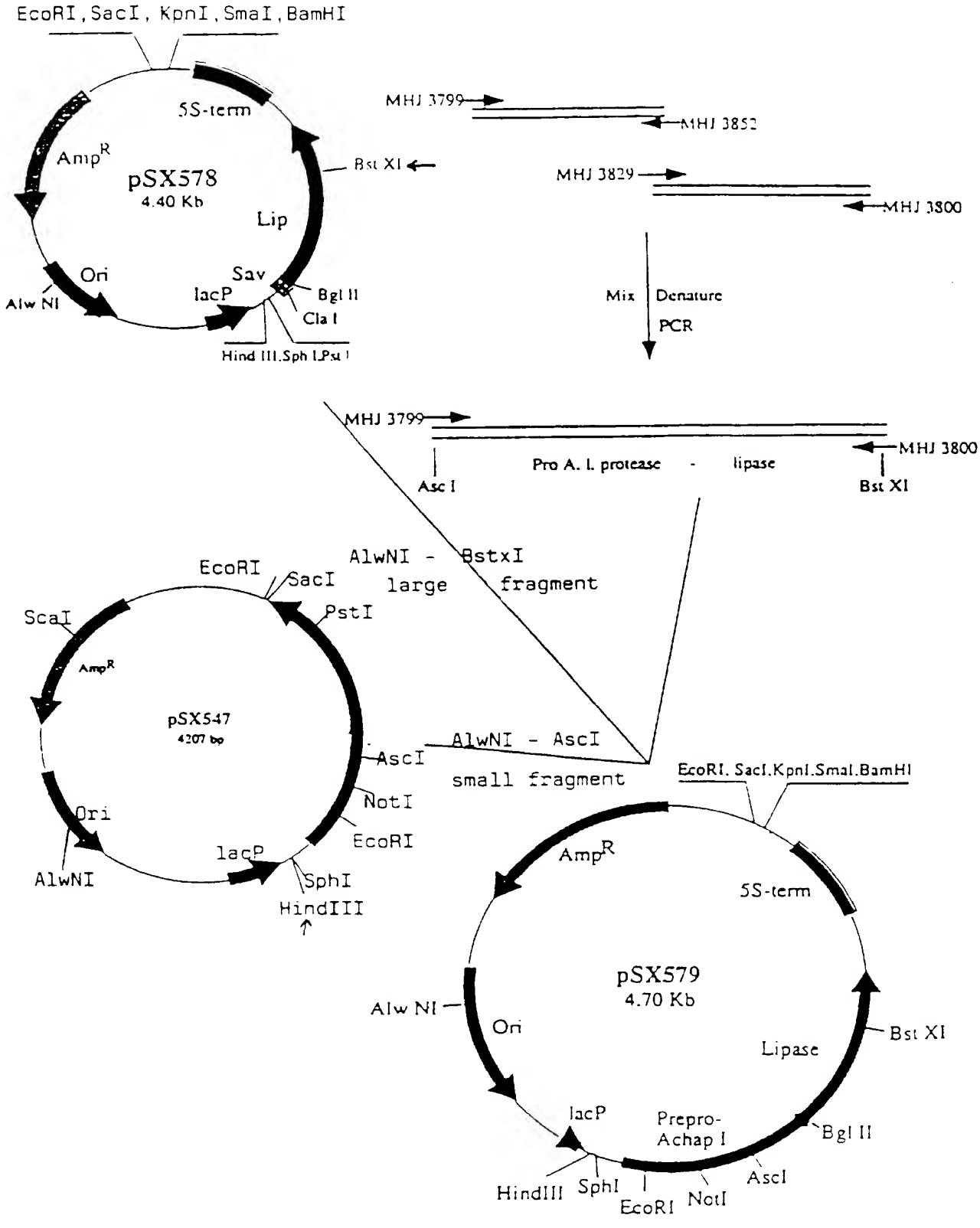
BstxI
Lip
Ori
Sav
lacP
Bgl II
Cla I
Hind III.Sph I.Pst I

Fig. 4b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5S-term
Amp^R
pSX578
4.40 Kb
Lip
Ori
Sav
lacP
Alw NI
Bgl II
Cla I
Hind III.Sph I.Pst I
Bst XI
MHJ 3799
MHJ 3852
MHJ 3829
MHJ 3800
Mix
Denature
PCR
MHJ 3799
MHJ 3800
Asc I
Pro A. l. protease - lipase
Bst XI
AlwNI - BstxI
large fragment
EcoRI
SacI
PstI
ScaI
Amp^R
pSX547
4207 bp
AscI
NotI
EcoRI
SphI
HindIII
Ori
AlwNI
lacP
AlwNI - AscI
small fragment
EcoRI. SacI.KpnI.SmaI.BamHI
Amp^R
5S-term
pSX579
4.70 Kb
Alw NI
Ori
Lipase
Bst XI
lacP
Prepro-
Achap I
Bgl II
HindIII
SphI
EcoRI
NotI
AscI

Fig. 4c

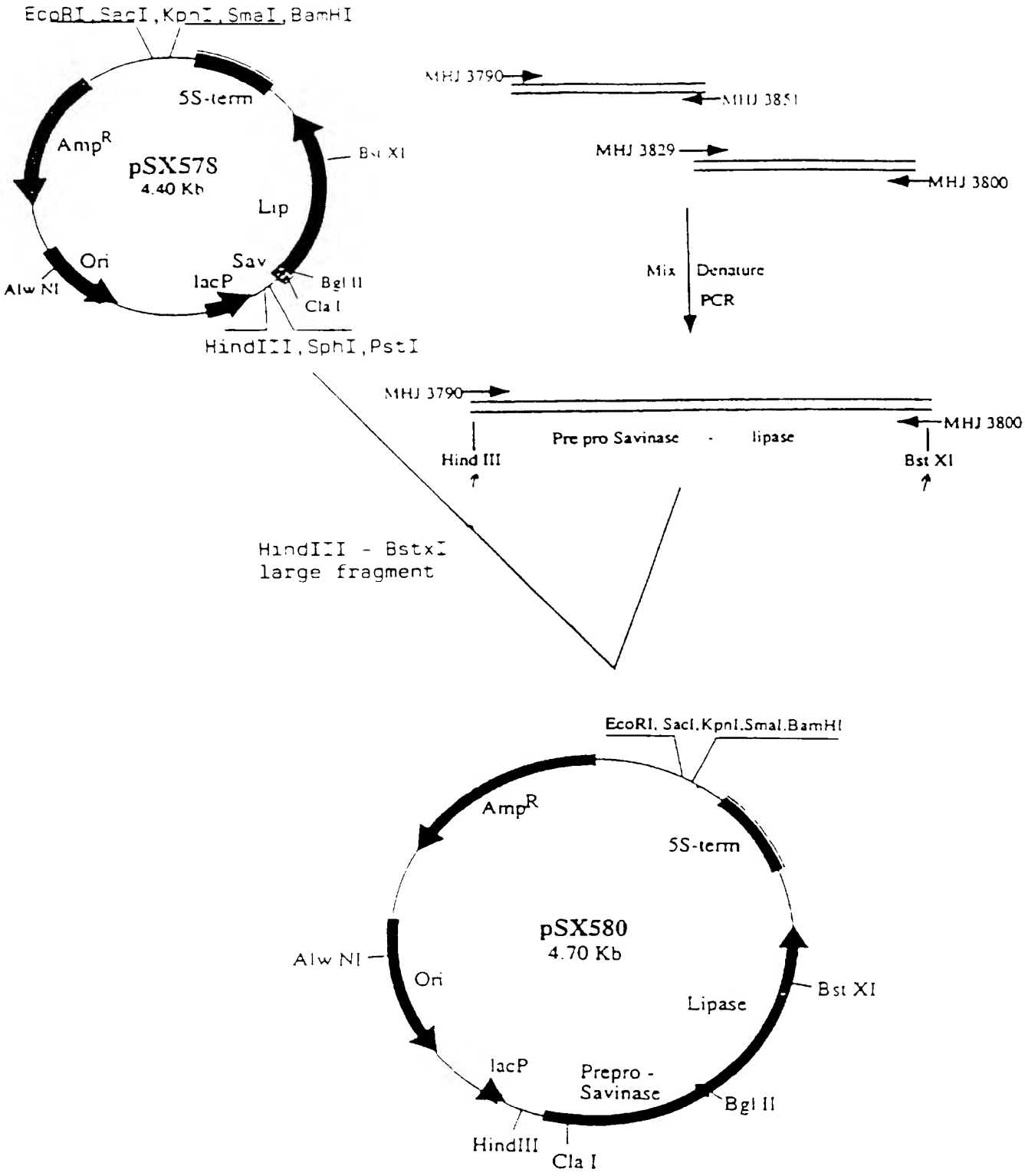
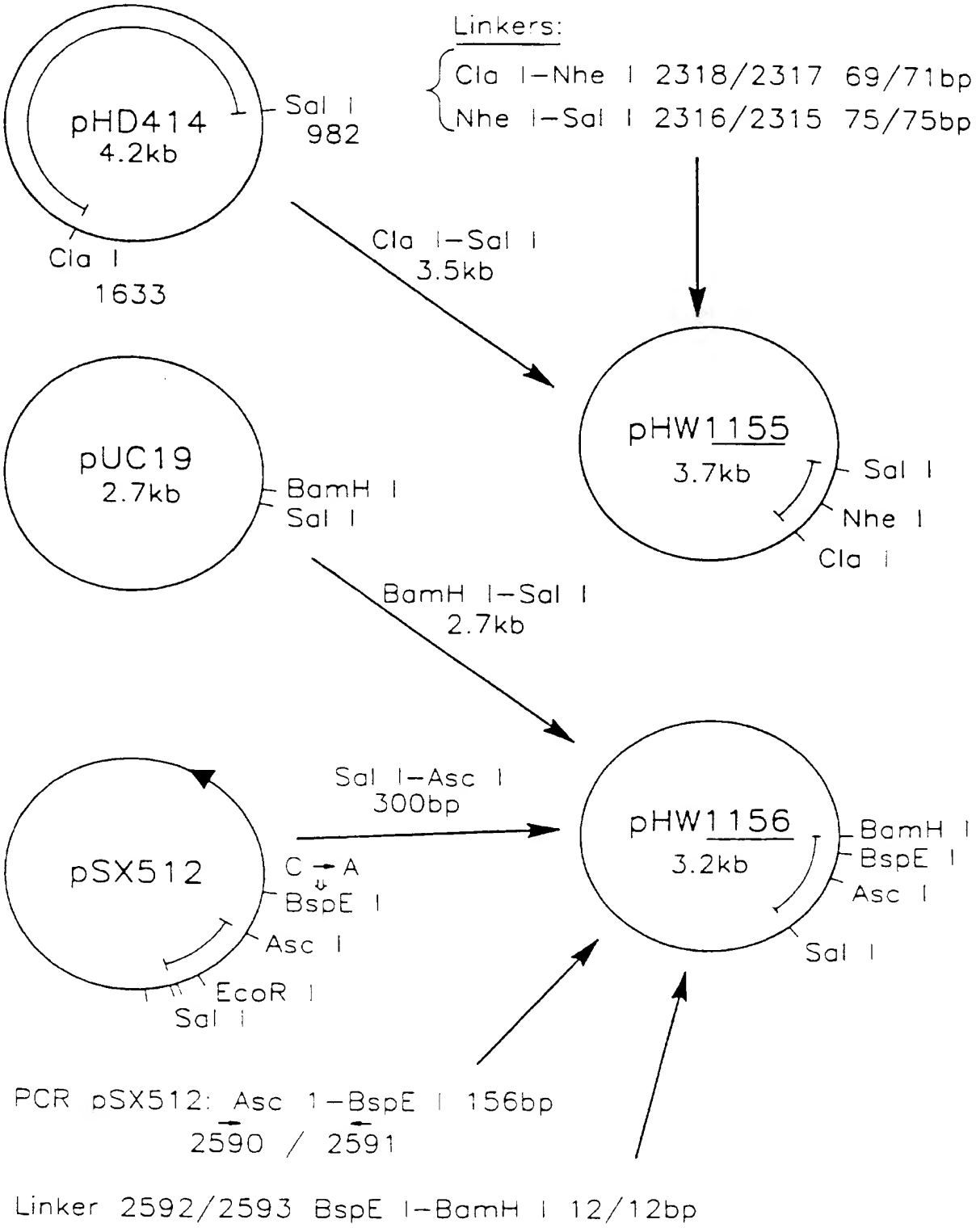

EcoRI, SacI, KpnI, SmaI, BamHI
5S-term
Amp^R
pSX578
4.40 Kb
Bst XI
Lip
Ori
Sav
AlwNI
lacP
Bgl II
Cla I
HindIII, SphI, PstI
MHJ 3790
MHJ 3851
MHJ 3829
MHJ 3800
Mix
Denature
PCR
MHJ 3790
MHJ 3800
Pre pro Savinase - lipase
Hind III
Bst XI
HindIII - BstxI
large fragment
EcoRI, SacI, KpnI, SmaI, BamHI
Amp^R
5S-term
pSX580
4.70 Kb
AlwNI
Ori
Lipase
Bst XI
lacP
Prepro - Savinase
Bgl II
HindIII
Cla I

Fig. 4d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Nhe I–Sal I 2316/2315 75/75bp
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Sal I
982
Cla I
1633
Cla I–Sal I
3.5kb
pHW1155
3.7kb
Sal I
Nhe I
Cla I
pUC19
2.7kb
BamH I
Sal I
BamH I–Sal I
2.7kb
Sal I–Asc I
300bp
pHW1156
3.2kb
BamH I
BspE I
Asc I
Sal I
pSX512
C → A
BspE I
Asc I
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Sal I
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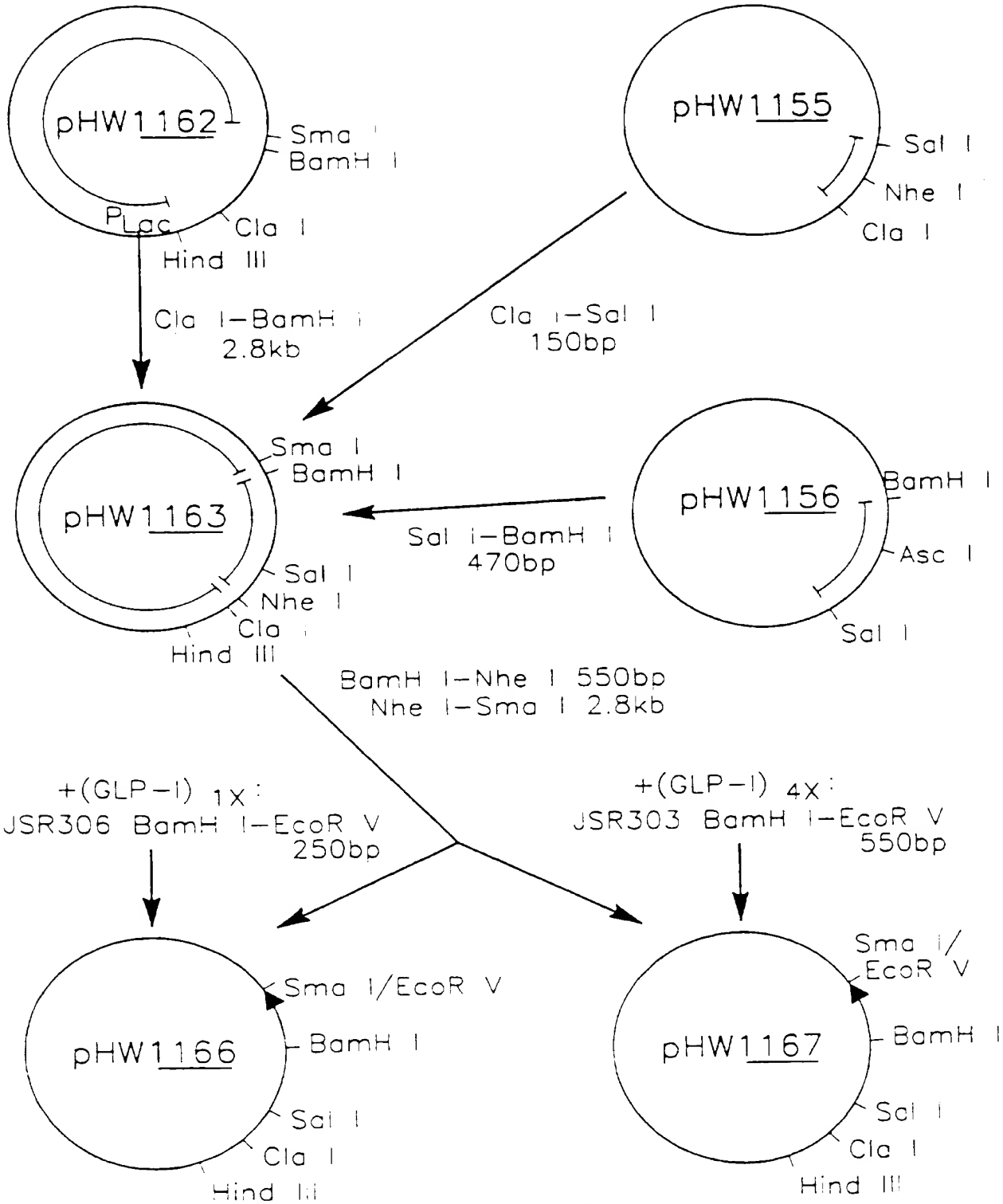
2590 / 2591
Linker 2592/2593 BspE I–BamH I 12/12bp

Fig. 5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BamH I
PLac
Cla I
Hind III
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Sal I
Nhe I
Cla I
Cla I–BamH I
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Cla I–Sal I
150bp
pHW1163
Sma I
BamH I
Sal I
Nhe I
Cla I
Hind III
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BamH I
Asc I
Sal I
Sal I–BamH I
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BamH I–Nhe I 550bp
Nhe I–Sma I 2.8kb
+(GLP-1) 1X:
JSR306 BamH I–EcoR V
250bp
+(GLP-1) 4X:
JSR303 BamH I–EcoR V
550bp
pHW1166
Sma I/EcoR V
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Sal I
Cla I
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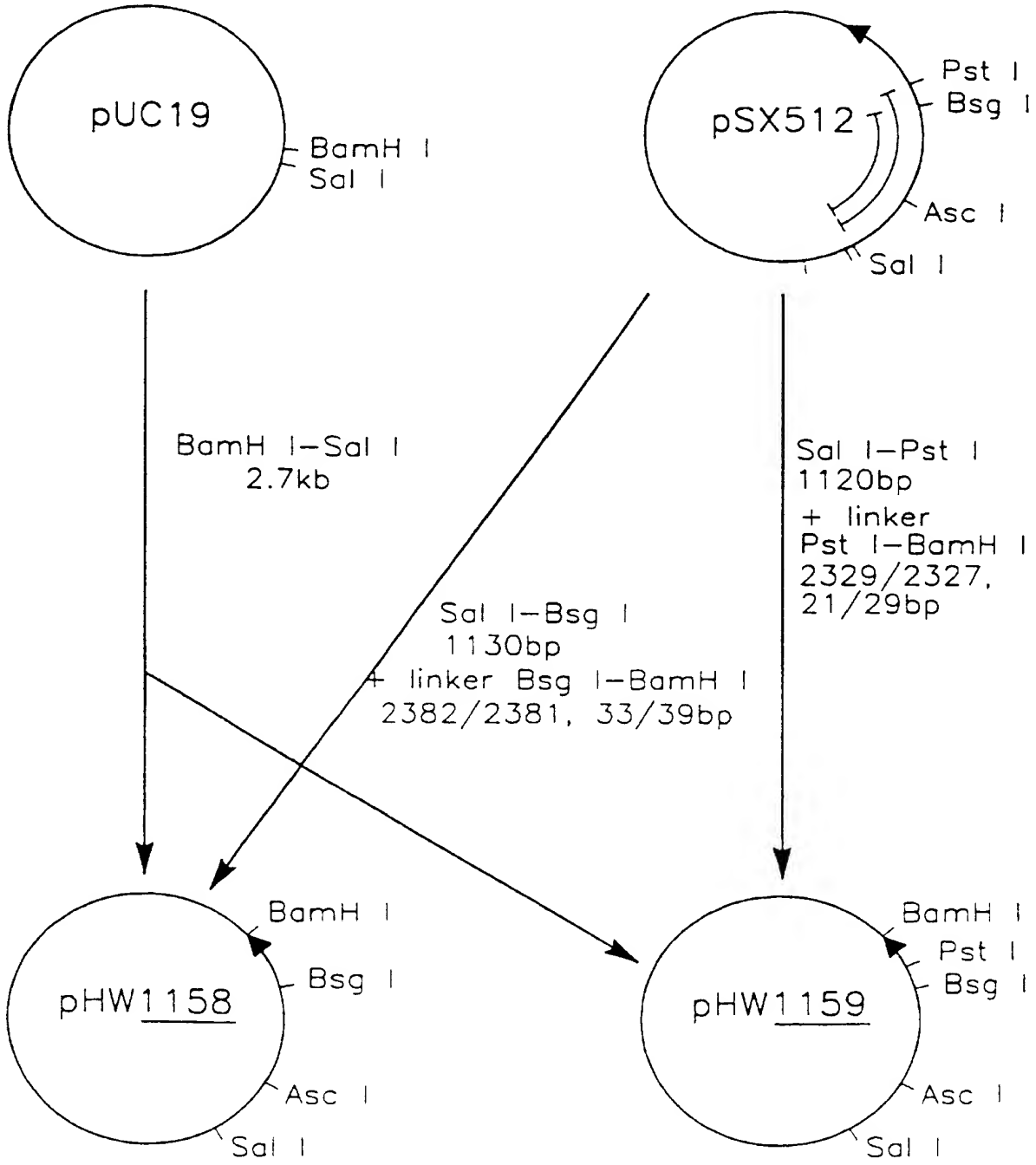
Sma I/
EcoR V
BamH I
Sal I
Cla I
Hind III

Fig. 6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BamH I
Sal I
pSX512
Pst I
Bsg I
Asc I
Sal I
BamH I–Sal I
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Sal I–Pst I
1120bp
+ linker
Pst I–BamH I
2329/2327,
21/29bp
Sal I–Bsg I
1130bp
+ linker Bsg I–BamH I
2382/2381, 33/39bp
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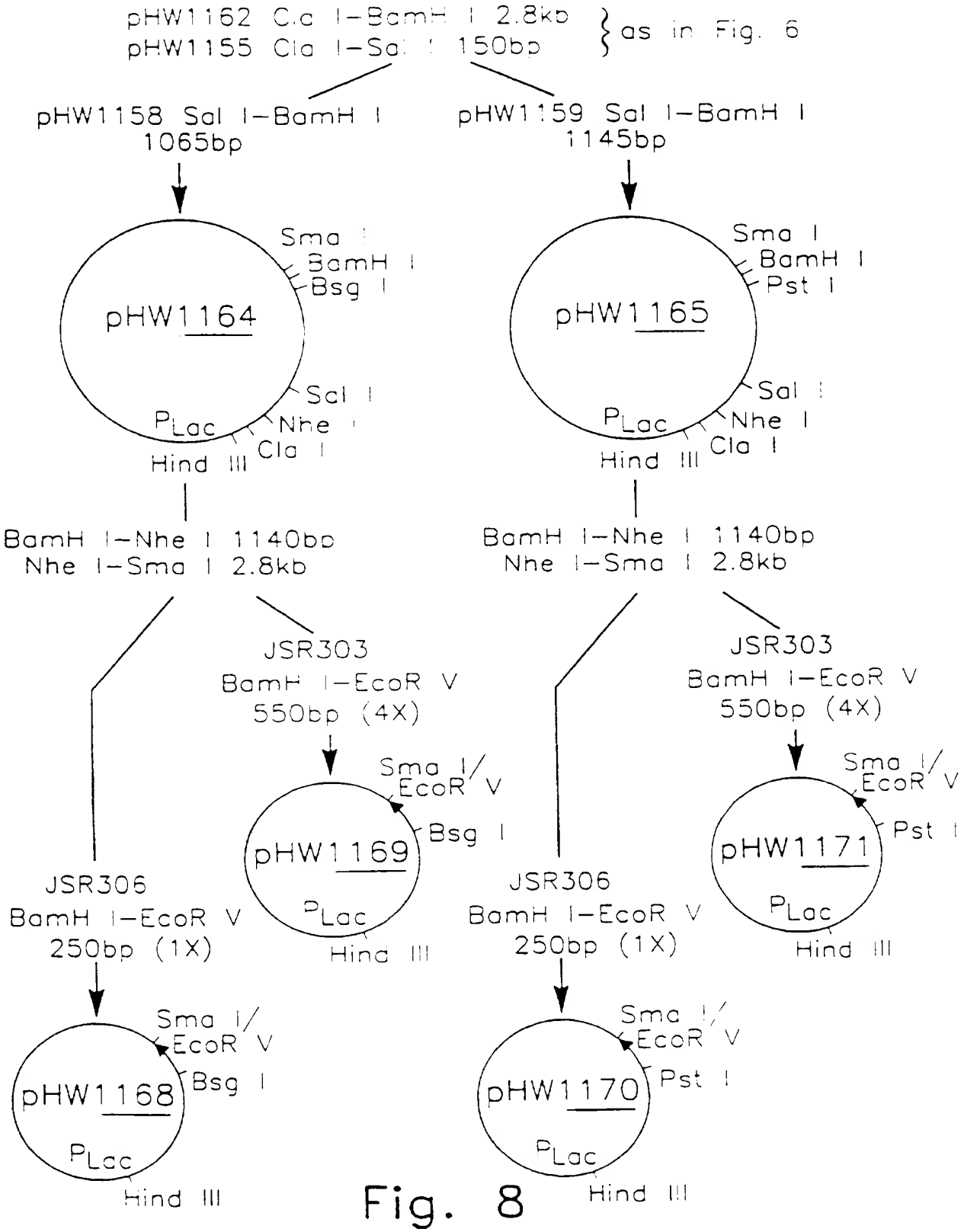
BamH I
Bsg I
Asc I
Sal I
pHW1159
BamH I
Pst I
Bsg I
Asc I
Sal I

Fig. 7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pHW1155 Cla I–Sal I 150bp
as in Fig. 6
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1065bp
pHW1159 Sal I–BamH I
1145bp
pHW1164
Sma I
BamH I
Bsg I
Sal I
PLac
Nhe I
Cla I
Hind III
pHW1165
Sma I
BamH I
Pst I
Sal I
PLac
Nhe I
Cla I
Hind III
BamH I–Nhe I 1140bp
Nhe I–Sma I 2.8kb
BamH I–Nhe I 1140bp
Nhe I–Sma I 2.8kb
JSR303
BamH I–EcoR V
550bp (4X)
JSR303
BamH I–EcoR V
550bp (4X)
Sma I/EcoR V
Bsg I
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PLac
Hind III
Sma I/EcoR V
Pst I
pHW1171
PLac
Hind III
JSR306
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JSR306
BamH I–EcoR V
250bp (1X)
Sma I/EcoR V
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Sma I/EcoR V
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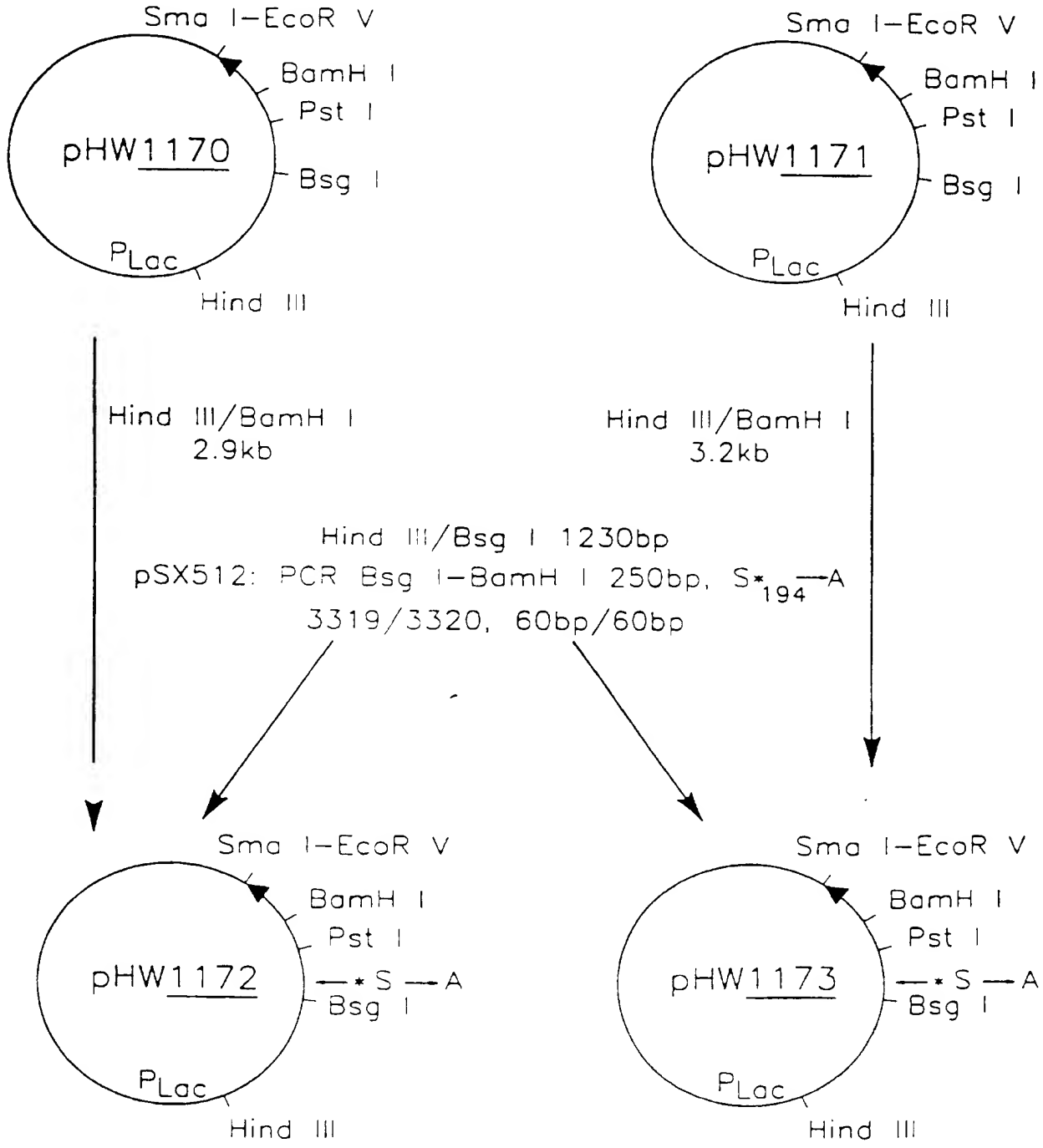
Hind III

Fig. 8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PLac
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Hind III/BamH I
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Hind III/BamH I
3.2kb
Hind III/Bsg I 1230bp
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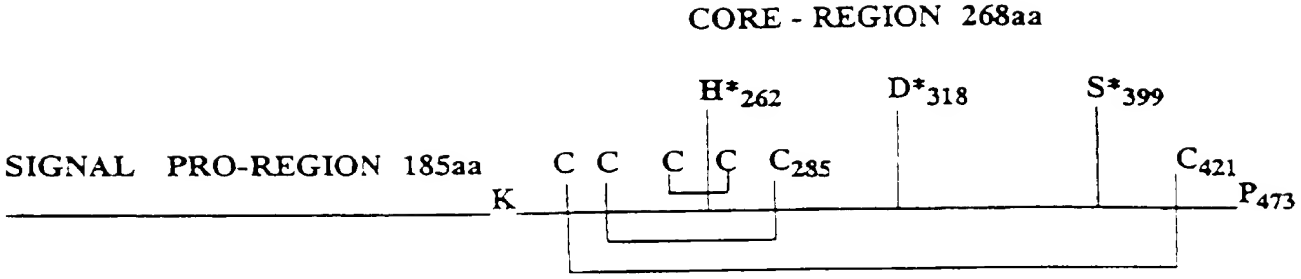
3319/3320, 60bp/60bp
Sma I–EcoR V
BamH I
Pst I
pHW1172
→* S → A
Bsg I
PLac
Hind III
Sma I–EcoR V
BamH I
Pst I
pHW1173
→* S → A
Bsg I
PLac
Hind III

Fig. 9



R_{205}

$Q_{413}R$

Δ 398–409 (S*) , max. 2 S-S

R_{429}

Δ 45 aa , max. 3 S-S

R_{473}

FULL LENGTH : $S^*_{194} \rightarrow A$

ALL FUSIONS ARE WITH GLP-1 7-36 R MONOMER AND TETRAMER

FIGURE 10

GENE STRUCTURE

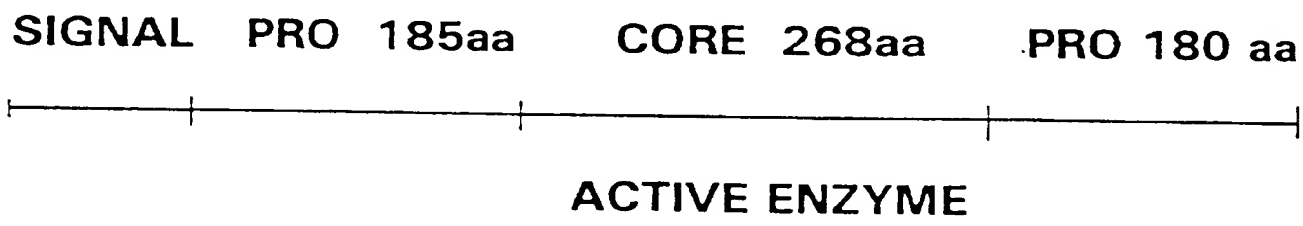


Fig. 11

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK 95/00498

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/70, C12N 15/74, C12N 15/62 // C12N 015/78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, DBA,CA SCISEARCH, WPI, EDOC, EPOQUE, PAJ, PCT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0387646 A1 (WAKO PURE CHEMICAL INDUSTRIES LTD), 19 Sept 1990 (19.09.90), the whole document	1-5,11-15, 19-20,24-26
A		6-10,16-18, 21-23

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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 1996	12 -04- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/02/96

International application No.
PCT/DK 95/00498

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0387646	19/09/90	JP-A- 2242686	27/09/90
		US-A- 5248599	28/09/93